

EXTENDED EXPERIMENTAL PROCEDURES**Electrophysiology****Hippocampal Cultures**

Primary cultured hippocampal neurons were prepared from P0 Sprague-Dawley rat pups. The CA1 and CA3 regions were isolated, digested with 0.4 mg/ml papain (Worthington, Lakewood, NJ, USA), and plated onto glass coverslips precoated with 1:30 Matrigel (Beckton Dickinson Labware, Bedford, MA, USA) at a density of 65,000/cm². Cultures were maintained in a 5% CO₂ humid incubator with Neurobasal-A medium (Invitrogen Carlsbad, CA, USA) containing 1.25% FBS (Hyclone, Logan, UT, USA), 4% B-27 supplement (GIBCO, Grand Island, NY, USA), 2 mM Glutamax (GIBCO), and FUDR (2 mg/ml, Sigma).

Calcium Phosphate Transfection

Six to ten days in vitro hippocampal neurons were grown at 65,000 cells/well in a 24-well plate. DNA/CaCl₂ mix for each well: 1.5–3 µg DNA (QIAGEN endotoxin-free preparation) + 1.875 µl 2 M CaCl₂ (final Ca²⁺ concentration 250 mM) in 15 µl total H₂O. To DNA/CaCl₂ was added 15 µl of 2X HEPES-buffered saline (pH 7.05), and the final volume was mixed well by pipetting. After 20 min at RT, the 30 µl DNA/CaCl₂/HBS mixture was dropped into each well (from which the growth medium had been temporarily removed and replaced with 400 µl warm MEM) and transfection allowed to proceed at 37°C for 45–60 min. Each well was then washed with 3 × 1 ml warm MEM and the growth medium replaced. Opsin expression was generally observed within 20–24 hr.

Electrophysiology

Whole-cell patch clamp recordings were performed as previously described (intracellular solution: 129 mM K-gluconate, 10 mM HEPES, 10 mM KCl, 4 mM MgATP, 0.3 mM Na₃GTP, titrated to pH 7.2; extracellular Tyrode: 125 mM NaCl, 2 mM KCl, 3 mM CaCl₂, 1 mM MgCl₂, 30 mM glucose, and 25 mM HEPES, titrated to pH 7.3). For voltage-clamp recordings, cells were held at –70 mV. Light was delivered from a 300W DG-4 lamp (Sutter Instruments, Novato, CA, USA) through a 593/40 nm filter (Semrock, Rochester, NY, USA) and a Leica 40X/0.8NA water objective; light power at the sample was 3 mW/mm². Whole-cell patch clamp data are from cultured hippocampal neurons either transfected or transduced with lentiviral eNpHR3.0 and eNpHR3.1 and allowed to express for one week. Expression was driven by the human CaMKIIα promoter and visualized by fusion to EYFP.

For electrophysiological measurement of continuous inhibition of evoked spiking by eNpHR3.1 in slice, mice were sacrificed and brains were sliced in a sucrose solution containing the following (in mM): 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄H₁₄O₇, .5 CaCl₂H₄O₂, 11 glucose, and 234 sucrose. aCSF contained (in mM) 126 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl, and 10 glucose. Patch electrodes were filled with the following (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 10 EGTA, and 2 MgCl (pH adjusted to 7.3 with KOH).

Measurement of Learning and Memory in the Fear Conditioning Paradigm

Mice were trained in a fear-conditioning apparatus that was modified to enable light delivery during training and/or testing, as described in the [Experimental Procedures](#) section as follows: In the first experiment ([Figure 2](#)) mice were trained and tested as follows: Day 1 training with continuous 561 nm light administration (light ON). Day 2 contextual and cued tests (2 hr apart) without light administration (light OFF). Day 3 training, light OFF. Day 4 test, light OFF. Day 5 contextual and cued tests, light ON. In the first remote memory experiment ([Figure 3A](#)): Day 1 training, light OFF. Day 29 contextual and cued tests, light ON. Day 30 test light OFF. In a second remote memory experiment ([Figure 3C](#)): Day 1 training, light OFF. Day 64 contextual test, light ON. In a third remote memory experiment ([Figure 3D](#)): Day 1 training, light OFF. Day 85 contextual test, light ON. In a fourth experiment ([Figure 4D](#)): Day 1 training, light OFF. Day 36 test, light OFF. Day 37 test light ON. Day 38 test with 3 min light OFF followed by 3 min light ON. In the BLA experiment ([Figures 2H and 2I](#)) mice were trained on day 1 with light ON and tested for contextual and cued fear on day 2 with light OFF. In the ACC ([Figures 7A and 7B](#)) and OB ([Figure S3](#)) experiments, mice were trained on day 1 with the light OFF, tested on day 2 with the light ON, and then tested on day 29 with light ON. For prolonged light exposure ([Figures 4A, 4B, and 7D](#)), the optical fibers were passed through the conditioning cage into a regular housing cage with bedding, and light was delivered in this cage for 30 min. The mouse was then placed in the conditioning cage for a 5 min test, as light delivery continued without interruption.

Neuronal Activation Imaging by c-Fos Staining

YFP control and eNpHR3.1 mice were trained with light administration during conditioning (without tone presentation) and sacrificed 90 min later to test for c-Fos levels (as described above). Two other groups of nontrained control and eNpHR3.1 mice were sacrificed from their home cages. For remote memory, YFP controls and eNpHR3.1 mice were fear conditioned without light, exposed to the conditioning context with light 28 days later, and sacrificed 90 min afterwards. The control groups at this time point were control and eNpHR3.1 mice that were trained, and then sacrificed from their home cages 28 days later without being re-exposed to the conditioning context. Statistical analysis was performed for mice (not slices) as the independent factor and corrected for multiple comparisons.

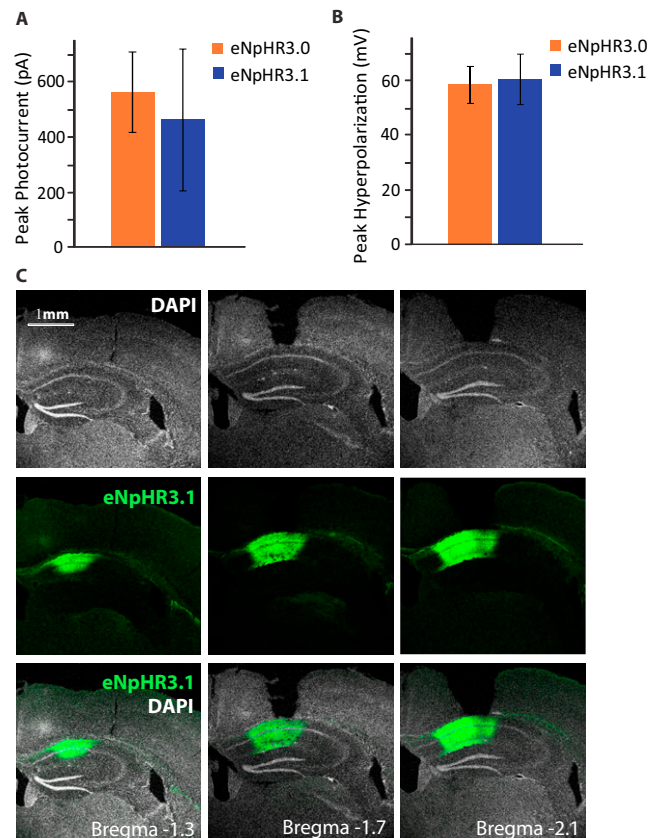


Figure S1. CA1 Specificity and Efficacy of eNpHR 3.1, Related to Figure 1

eNpHR3.1 is a truncated version of eNpHR3.0 with a deletion of the intrinsic N-terminal signal peptide that has comparable effects on membrane potential. Whole-cell patch clamp recordings (eNpHR3.0, $n = 10$ cells; eNpHR3.1, $n = 4$ cells) show similar photocurrents (A) and resulting hyperpolarizations (B) (average \pm SEM, $p > 0.5$ for both).

(C) The eNpHR3.1 protein is expressed in CA1, but under these expression conditions not in other hippocampal subfields, in the parietal cortex above the injection sites, in thalamus, or in habenula. The cannula track (at bregma -1.94) can be seen above the expression sites. The volume of infection covers a substantial fraction of dorsal CA1 (0.875 ± 0.05 mm³; $n = 12$ mice).

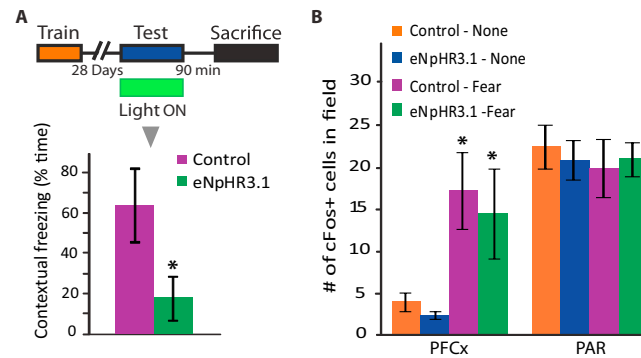


Figure S2. Expanded Information on Brain-wide Mapping of Circuit Activity Controlled by the Hippocampus during Remote Recall, Related to Figure 6

(A) CA1 optogenetic inhibition prevented recall of remote memory that was acquired 28 days earlier, with no tone presentation during conditioning. ($p < 0.05$; control, $n = 3$, $61.0\% \pm 17.6\%$ freezing; eNpHR3.1, $n = 3$, $17.1\% \pm 10.5\%$ freezing). Mice were sacrificed 90 min after conditioning, and brains were stained for c-Fos.

(B) Remote recall 28 days following conditioning resulted in a significant increase in prefrontal cortex (PFCx) c-Fos expression in both control and eNpHR3.1 mice ($p < 0.05$ for both). No changes were observed in c-Fos levels in parietal cortex (PAR).

Data presented as mean \pm SEM.

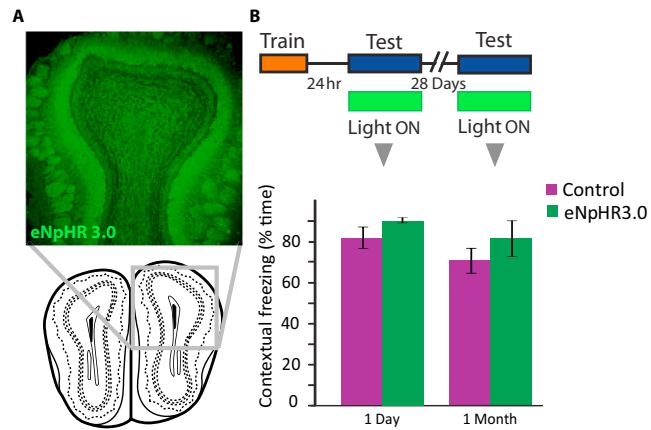


Figure S3. Olfactory Bulb Optogenetic Inhibition Does Not Disrupt Recent or Remote Fear Recall, Related to Figure 7

eNpHR3.0 expression in the olfactory bulbs (OB, panel A) does not affect either recent (control, $n = 11$, $81.44\% \pm 5.0\%$ freezing; eNpHR3.0, $n = 8$, $90.5\% \pm 1.2\%$ freezing; $p > 0.5$) or remote ($71.37\% \pm 6.11\%$ versus $82.25\% \pm 8.7\%$ freezing) fear recall (panel B) upon light administration.